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Short Report: Diagnostic Testing for Hemorrhagic Fevers in Pakistan: 2007–2013

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Abstract. Crimean–Congo hemorrhagic fever virus (CCHFV) and dengue virus (DENV) are endemic to Pakistan. Patients presenting with symptoms of fever, bleeding, and rash cannot be distinguished without appropriate testing. We report data on 354 samples tested for CCHFV at The Aga Khan University Hospital in Pakistan between 2007 and 2013. All samples were tested for the presence of CCHFV RNA. Some samples were also tested for DENV RNA, NS-1 antigen, and/or reactive immunoglobulin M antibodies. Of 354 clinical specimens screened for CCHFV, 52 specimens were positive, with 24 cases in 2013 alone. Most cases were from Sindh and Baluchistan, which border other CCHFV-endemic regions: Iran and Afghanistan. Among CCHFV-negative samples, 168 samples were tested for DENV, and 36% of these samples were found to be DENV-positive. Rapid differentiation of CCHFV and DENV can prevent nosocomial transmission and result in time and cost savings for patients and healthcare workers.

Crimean–Congo hemorrhagic fever (CCHF) is endemic to Pakistan.[1] The *Hyalomma* tick is the principal vector for CCHF virus (CCHFV), and conditions affecting its lifecycle and distribution contribute to CCHF expansion. Severe disease only occurs in humans, who usually acquire the virus through a bite by an infected tick or direct contact with blood or tissues of a viremic animal or patient.

The viral incubation period of 2–9 days is generally followed by progressive bruising and severe bleeding, whereby the patient becomes highly infectious. The general case fatality rate (CFR) of CCHF in Pakistan is up to 50%; however, nosocomial transmission with a CFR of up to 90% has been reported.[2,3]

The main risk area for CCHF in Pakistan is the southwest province of Baluchistan, a primarily desert region that borders CCHFV-endemic regions of Afghanistan and Iran.[4] The first major CCHF outbreak coincided with the peak of the 1997–2002 droughts in Baluchistan and Sindh in the year 2000.[5] Reports indicate a biannual surge in CCHFV rates (from March to May and from August to October) that coincides with a hot and dry season, which is linked to prevalence of ticks.[4] Cases of CCHF in Pakistan are also associated with the transport of animals to cities.[6] In addition, the propensity of CCHF to spread nosocomially has led to outbreaks involving healthcare workers.[7]

Dengue, an acute febrile illness associated with rash and arthralgia caused by a virus classified with the family *Flaviviridae*, genus *Flavivirus* (dengue virus [DENV]), also circulates in Pakistan. Although human cases of dengue are typically mild and self-limiting, a severe form of the disease associated with thrombocytopenia and hemorrhagic manifestations is also present (dengue hemorrhagic fever [DHF]). Since the first recorded outbreak of dengue in Pakistan in 1994, there has been an exponential increase in the cases causing significant morbidity and mortality.[7,8] All four major genotypes of DENV have been identified in Pakistan; genotype 3 is the most predominant serotype currently circulating in Pakistan followed by genotypes 1 and 2, with genotype 4 being the least common.[9] Given the similarity in early clinical presentations of CCHF and DHF, testing for both CCHFV and DENV is essential for differential diagnosis of patients with hemorrhagic symptoms.

Here, we report the laboratory diagnosis of clinical specimens received for CCHFV testing at The Aga Khan University Hospital Laboratory during 2007–2013.

This work received approval from the Ethical Review Committee of The Aga Khan University in Pakistan.

Samples were received at The Aga Khan University Hospital Clinical Laboratory from its network of 200 collection points located throughout Pakistan. Whole-blood samples were collected from patients with hemorrhagic manifestations (current or subsided) and a clinical disease course consistent with CCHF. Serum separated from blood was stored at –80°C and transported to Karachi on dry ice for testing.

The requisite data collection form included demographic information, clinical history pertaining to primary symptoms, such as fever and bleeding, and contact with cattle, animal skins, or CCHFV-infected individuals. The patient data were limited to the information provided at the time of sample collection, because patient contact and/or follow-up were typically not possible because of lack of relevant information. Unfortunately, in many cases, data regarding occupation, possible tick bites, and other possible contact leading to infection were missing.

RNA was extracted from 150 μL serum using the Nucleospin Kit (Macherey-Nagel, GmbH & Co. KG). From 2007 to 2011, a nested polymerase chain reaction (PCR) method targeting the small (S) segment of the CCHFV genome was used to detect CCHFV RNA as described previously.[10] From 2011 to 2013, detection of CCHFV was performed using a real-time reverse transcription PCR (RT-PCR) assay for detection of a broad range of CCHFV genotypes as previously described.[11] In brief, the assay was run on the Light Cycler 2.0 System (Roche Molecular Systems Inc., Branchburg, NJ) and used a synthetic positive DNA control and an assay cutoff of Ct (threshold cycle) 39, which is equivalent to five genome copies.

Serum samples were tested for DENV immunoglobulin M (IgM) by enzyme-linked immunosorbent assay (ELISA) assay (PanBio, Inverness Medical Innovation, Queensland, Australia). DENV NS1 antigen was detected using an immunochromatographic test (PanBio). DENV RNA was detected using a one-step RT-PCR assay specific for the 3′ noncoding region of the DENV genome as previously described.[12]

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TABLE 1
Testing for CCHFV and DENV, 2007–2013

Year	Samples tested for CCHFV	CCHF PCR-positive (%)	Samples tested for DENV*	DENV-positive (%)†	Confirmed VHF (%)‡
2007	24	0	17	2 (11)	2 (8.3)
2008	11	0	7	0 (0)	0 (0)
2009	33	3 (9.1)	13	8 (62.5)	11 (33.3)
2010	62	7 (11.3)	23	16 (69.6)	23 (37.1)
2011	35	5 (14.3)	15	2 (13.3)	7 (20.0)
2012	58	13 (22.4)	32	8 (25.0)	21 (36.2)
2013	131	24 (18.3)	61	25 (41.0)	49 (37.4)
Total	354	52 (14.7)	168	61 (36.3)	113 (31.9)

*CCHF-negative samples.
†Percentage positive for DENV refers to the number of positives over the total CCHF-negative samples on which DENV testing was performed (IgM/NS1 antigen/DENV PCR).
‡Total number of confirmed VHF viruses (CCHFV and DENV) identified from samples sent for CCHF testing.
VHF = viral hemorrhagic fever.

In total, 354 specimens were received for CCHFV testing from 2007 to 2013 (Table 1). Fifty-two patients were found to be CCHFV PCR-positive, with an average Ct value of 25 (SD = 6). The median age of patients was 34 years old, and the gender ratio of positive samples was approximately 5:1, with 46 positive cases diagnosed in males and 6 positive cases diagnosed in females (data not shown).

The number of laboratory-diagnosed CCHFV-positive cases increased annually from 2007 onward, and most of the cases were observed in 2013 (24 of 52). Also, in 2013, two times as many samples were received for CCHFV PCR testing, and 18% of these samples tested positive. This may represent an increase in patients presenting with hemorrhagic manifestations or an increased awareness of this testing facility.

Monthwise analysis of CCHFV tests showed two surges in incidence: one surge between April and July and one surge between October and December (Figure 1A). CCHF cases in Pakistan were from all four provinces: Sindh (N = 25), Baluchistan (N = 12), Punjab (N = 4), and Khyber-Pakhtunkhwa Province (KPK; N = 11) (Figure 1B). The highest number of positive cases was received from the city of Karachi (possibly because of the location of the test facility) followed by Quetta and Peshawar. Occupational

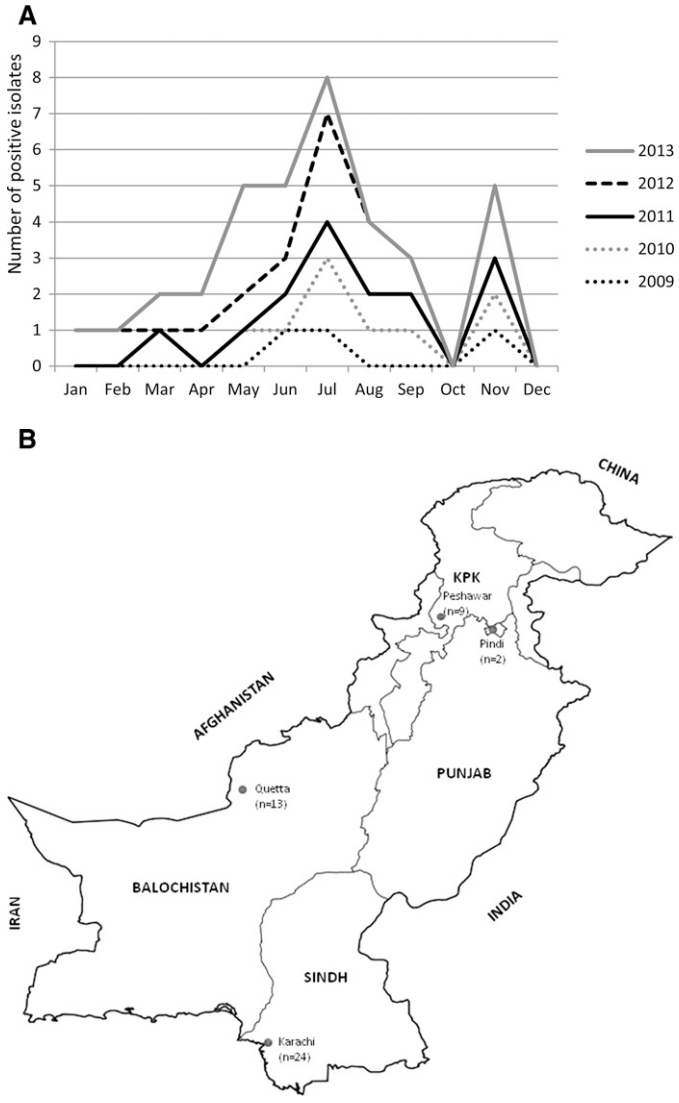

FIGURE 1. Pattern of CCHFV-positive cases diagnosed (2007–2013). (**A**) The graph shows the monthwise trend of CCHFV PCR-positive cases diagnosed each year. (**B**) The map of Pakistan illustrates provinces (Baluchistan, Sindh, Punjab, and KPK) and cities from which CCHFV-positive specimens were received (numbers in parentheses).

information was available for only 18 CCHFV-positive patients, of which 8 patients reported contact with animals. The patients belonged to different demographic groups, and many were not typically associated with an at-risk profession for exposure to CCHFV: butcher ($N = 3$), housewife ($N = 3$), animal husbandry ($N = 2$), waiter ($N = 2$), leather factory worker ($N = 1$), laborer ($N = 1$), fisherman ($N = 1$), surgeon ($N = 1$), college student ($N = 1$), businessman ($N = 1$), cloth factory worker ($N = 1$), and shopkeeper ($N = 1$).

In the past, the Balochistan province and its surrounding regions of Iran and Afghanistan have been found to be highly endemic for CCHF.[4] However, in 2013, a rise in cases (Figure 1A) was associated with an outbreak in KPK, where samples from 17 CCHF-suspected cases were received from Peshawar and Waziristan, northern KPK, which is situated by the Afghanistan border. Eight of these cases were CCHFV PCR-positive. During the same period, four clinical specimens received from Punjab tested positive for CCHFV; the province has seldom been associated with CCHF.

Among 302 CCHF-negative samples, 168 samples were tested for DENV (Table 1); 61 (36%) patients were positive for DENV infection: 4 by PCR and 57 by serology-based testing.

This study highlights the differences in etiologies for cases of viral hemorrhagic diseases in Pakistan. Of 113 cases with an established cause tested between 2007 and 2013, 52 cases were determined to be CCHF, and 61 cases were determined to be DHF. The ability to distinguish these viruses is critical in the management of patients. Nosocomial infection is associated with cases of CCHF, but this route of infection is not associated with hemorrhagic forms of dengue. The increase in CCHFV-positive specimens tested at The Aga Khan University Hospital in 2013 corroborates the rising trend in CCHF as reported by the World Health Organization (WHO), which documented 77 CCHF cases between January and September from all over Pakistan.[13] It is apparent that CCHF is a persistent arboviral zoonosis in Pakistan, with cases reported in each of the past 5 years. The apparent increase in the number of CCHF cases detected annually could represent a true increase in incidence of disease or may be an artefact resulting from increased awareness of this disease across the country. Overall, the trend of CCHF cases among males and those in contact with animals or animal skins is consistent with previous reports. However, a large proportion of cases was from the urban centers of Karachi and Quetta and included individuals in professions that are not typically associated with risk for CCHFV. These data suggest the possibility of indigenous transmission of CCHFV in these cities. Additional investigations are required to elucidate either the true route of exposure in urban centers or alternatively, the risk of contracting CCHF in an urban environment, such as the risk posed by animal butchery.

There is potential for human disease caused by other arboviruses circulating in Pakistan; inpatient data from The Aga Khan University Hospital show 102 patients suffering from fever and thrombocytopenia with no evidence of infection with either DENV or CCHFV (unpublished data). This suggests an alternative etiology; previous reports have found evidence of human exposure to other pathogenic arboviruses, including Chikungunya, Japanese encephalitis, and West Nile viruses, in Pakistan.[14] Additionally, hantaviruses and bacterial diseases, like leptospirosis and rickettsiosis, may also cause similar clinical symptoms.[15] Additional research studies should be undertaken to elucidate the potential burden of disease from non-hemorrhagic human pathogens that may be resulting severe cases of similar clinical disease.

In a region where DENV infection has become highly prevalent and cases of CCHF are also increasing, the availability of prompt and sensitive real-time RT-PCR assays enables rapid differentiation between CCHF and dengue fever. This is particularly important because of the serious consequences for both the patient and the healthcare facility if the diagnosis of CCHF is missed.

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